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Malignant Conversion and Metastasis of Mouse Skin Tumors: A Comparison of SENCAR and CD-1 Mice

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The progression of papillomas to squamous cell carcinomas (malignant conversion) was studied in the skin of SENCAR and Charles River CD-1 mice, using a three-stage treatment protocol. After initiation with 7,12-dimethylbenz(a)anthracene (DMBA) (stage 1) and limited promotion by 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (stage II), papilloma-bearing mice were treated (stage III) with either tumor initiators, such as urethane, *N*-methyl-*N*'nitro-*N*-nitrosoguanidine (MNNG) or 4-nitroquinoline-*n*-oxide (R-NQO), the promoter TPA, or solvent (acetone). Similar final carcinoma yields were found in the mice treated in stage III with TPA or acetone, although carcinomas developed earlier in the TPA-treated mice. In contrast, treatment with tumor initiators in stage III increased both the rate of appearance and the final yield of carcinomas. Similar results were obtained in both SENCAR and CD-1 mice. A papilloma stage appears to be necessary for carcinoma development since elimination of TPA treatment in stage II greatly reduced the incidence of both papillomas and carcinomas in both stocks of mice. The heterogeneity of papillomas with regard to progression to carcinomas is demonstrated by the low rate of conversion of TPA-dependent papillomas and the high rate of conversion of persistent papillomas in CD-1 mice. The carcinomas that develop using the three-stage regimen vary in metastatic potential. In CD-1 mice, the frequency of metastases to lymph nodes were similar in groups treated in stage III with MNNG, urethane, 4-NQO, TPA, or acetone, but treatment with urethane substantially increased metastases to the lung. In SENCAR mice, this effect of urethane was not observed, but lymph node and lung metastases appeared to be increased by stage III treatment with MNNG.

Introduction

The initiation-promotion model of epidermal carcinogenesis in mice has provided the basis for many conceptual advances in the understanding of the biology of carcinogenesis (*1–3*). Initiation by a single treatment with a low dose of carcinogen such as 7,12-dimethylbenz(a)anthracene (DMBA) (stage I) results in the permanent alteration of some epidermal cells (Table 1). In the absence of further treatment, these initiated cells do not develop into tumors, but the cellular changes are heritable. As a result of subsequent repeated exposure of initiated skin to promoting agents (stage II), multiple benign papillomas develop. The promotion process is reversible; papillomas do not develop after insufficient promoter treatment or if the interval between treatments is prolonged. Many papillomas regress when the promoting stimulus is removed (*4*). Other papillomas progress irreversibly to carcinomas, either spontaneously or as a result of further exposure to a genotoxic tumor initiator; this process has been termed malignant conversion (stage III) (*5*). Continued treatment with the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) increases the rate of malignant conversion, but does not alter the final carcinoma incidence (*5*). The factors responsible for this conversion have only been studied recently, although Berenblum (*6*) recognized the conversion of benign papillomas to carcinomas as a specific stage nearly 45 years ago. Metastasis of skin squamous cell carcinomas to the lungs and lymph nodes occurs as a fourth stage in epidermal carcinogenesis.

The irreversibility of both initiation and malignant conversion and the genotoxic nature of the active agents suggests that these processes involve genetic alterations. The increased proliferation rate in promoter-treated epidermis and in papillomas may increase the probability of genetic changes necessary for the progression of papillomas to carcinomas (*7,8*). Three-stage carcinogenesis has been demonstrated only in SENCAR

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Table 1. Multistage epidermal carcinogenesis.

Stage	End point
I. Initiation	Initiated cells
II. Promotion (stages 1 and 2)	Papillomas
III. Malignant conversion	Squamous cell carcinomas
IV. Metastasis	Metastatic squamous cell carcinomas

mice (*5*). In this report, results of further experiments with SENCAR mice are compared to those from similar experiments in Charles River CD-1 mice. Furthermore, rates and sites of metastasis of squamous cell carcinomas are compared in the two stocks of mice.

Materials and Methods

Chemicals

7,12-Dimethylbenz(a)anthracene (DMBA) was obtained from Eastman (Rochester, NY). 12-O-Tetradecanoylphorbol-13-acetate (TPA) was purchased from LC Services (Woburn, MA); *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was purchased from Aldrich (Milwaukee, WI); 4-nitroquinoline-*N*-oxide (4-NQO) was purchased from Sigma (St. Louis, MO); urethane was purchased from MCB (Cincinnati, OH).

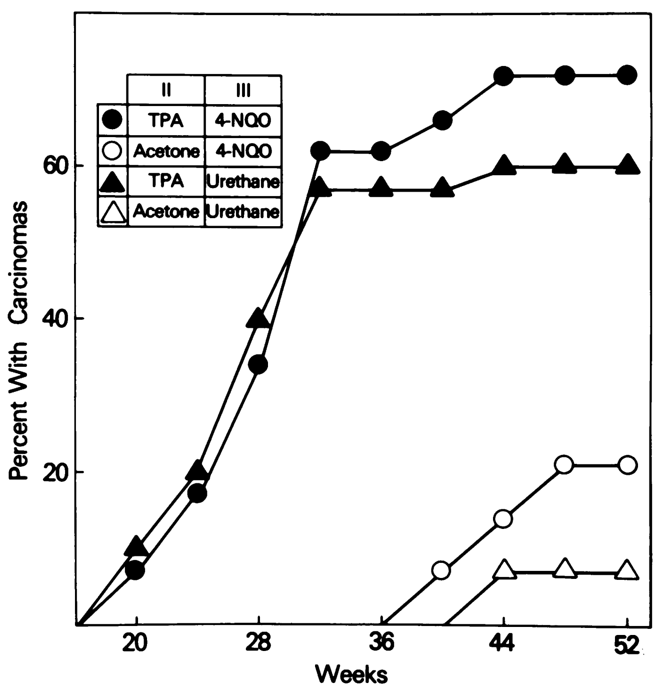

FIGURE 1. Carcinoma incidence in three-stage carcinogenesis in SENCAR mice. Groups of 30 female SENCAR mice were initiated with 20 μg DMBA (stage I) and treated with 10 weekly applications of either (●, ▲) TPA (2.0 μg/0.2 mL acetone)or (○, △) acetone in stage II. Once weekly treatments with (△, ▲) urethane (20 mg intraperitoneal) or (○,●) 4-NQO (250 μg/0.2 mL acetone) continued from weeks 11 to 40 in stage III. Suspected carcinomas were scored biweekly and were verified by histological examination.

Mice

SENCAR female mice were obtained from the NCI-DCT Animal Program. Charles River CD-1 female mice were purchased from Charles River Laboratories, Kingston, NY. Mice were shipped at 4 to 5 weeks of age.

Tumor Induction Experiments (Three-Stage Carcinogenesis)

Mice, 7 to 8 weeks old, were shaved before stage I initiation with a single topical application of DMBA. The DMBA dose was 20 μg in SENCAR mice and 50 μg in CD-1 mice. Stage II TPA treatments began one week after initiation. In SENCAR mice, 2 μg or 2.5 μg TPA was given weekly for 10 weeks; in CD-1 mice, 10 μg TPA was given weekly for 12 weeks. Stage III treatments with either urethane, 4-NQO, MNNG, TPA, or acetone were begun one week after Stage II treatments were completed. The duration of the once per week stage III treatments was 30 weeks in SENCAR mice and 40 weeks in CD-1 mice. All chemicals for topical treatment were dissolved in reagent-grade acetone (Baker, Philipsburg, NJ) and applied in a volume of 0.2 mL. Specific treatment protocols are described in the legends to figures.

Papilloma and carcinoma counts were recorded weekly or biweekly, and mice were weighed and shaved carefully once per month. A lesion was counted as a papilloma when it reached a diameter of more than 1 mm and was present for two consecutive weeks. Suspected carcinomas were verified histologically. Pathological evaluation of most papillomas was not performed; thus, early carcinomas arising in papillomas would not have been detected and the carcinoma incidences shown may underestimate the actual incidence.

Results and Discussion

Malignant Conversion in SENCAR mice

In tumor induction experiments in Swiss mice, most squamous cell carcinomas progress from papillomas (*9,10*), but the rate of conversion is low. The factors necessary for this malignant conversion have not been defined. After DMBA initiation (stage I) and limited TPA promotion (stage II) in SENCAR mice, continued TPA treatment of these papilloma-bearing mice was compared to repeated exposure to an initiator for the effect on carcinoma development (stage III). Exposure to the promoter TPA in stage III does not influence the final carcinoma yield, but the treatment of papilloma-bearing mice with genotoxic tumor initiators markedly accelerates and enhances malignant conversion. The carcinoma incidence was increased significantly by stage III treatments with 4-NQO, MNNG, and urethane for

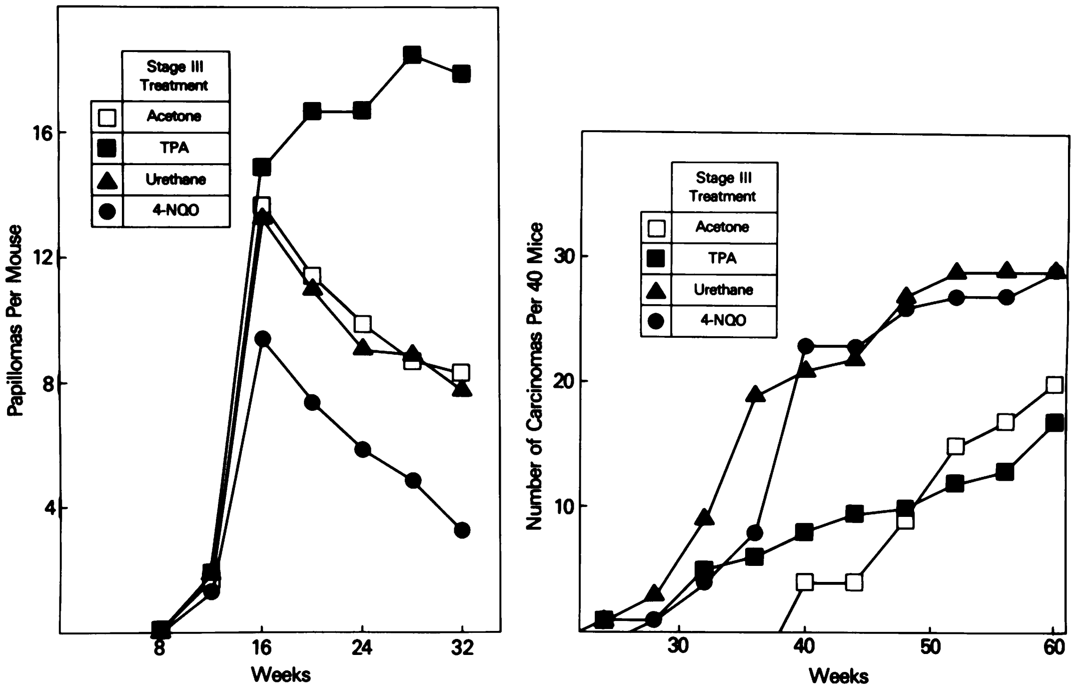

FIGURE 2. Papilloma and carcinoma incidence in a three-stage carcinogenesis experiment in CD-1 mice. Groups of 40 female CD-1 mice were initiated with 50 μg DMBA (stage I) and promoted with 12 weekly applications of 10 μg TPA (stage II). The indicated stage III treatments once weekly from weeks 13 to 52 were as follows: (□) acetone, 0.2 mL; (■) TPA, 10 μg; (▲) urethane, 20 mg IP; (●) 4-NQO, 250 μ.

30 weeks (*5*). Control groups of SENCAR mice treated only with the active stage III agents showed that urethane induced no tumors, 4-NQO induced few papillomas and no carcinomas, while MNNG was moderately active as a complete carcinogen (*5*).

When this experiment was repeated, stage III 4-NQO or urethane treatment of papilloma-bearing mice for 30 weeks again induced carcinomas in more than half the mice within 30 weeks (Fig. 1). In control groups treated with acetone rather than TPA in stage II, stage III treatment with 4-NQO or urethane induced few carcinomas (Fig. 1). These carcinomas appeared 20 weeks or more later than those in mice treated with TPA in stage II. In contrast, with MNNG in stage III, a high carcinoma yield was obtained even when TPA promotion in stage II was omitted (data not shown). Thus, MNNG is not useful as a stage III agent when given for 30 weeks. Fewer exposures to MNNG, over a period from 1 to 10 weeks, are currently being tested for stage III activity.

Malignant Conversion in Charles River CD-1 Mice

To verify the three-stage carcinogenesis results in mice other than SENCARs, a similar experiment was performed with Charles River CD-1 mice. In CD-1 mice initiated with DMBA (stage I) and promoted with TPA for 12 weeks (stage II), many papillomas regressed after TPA exposure was terminated (Fig. 2*a*). In the group in which TPA was continued, the papilloma incidence continued to rise until week 28. However, the final carcinoma yield was essentially equal in groups receiving continuous TPA or acetone solvent alone in stage III (Fig. 2*b*). Since the number of papillomas remaining at the time carcinomas were first seen was markedly reduced in the acetone group, TPA-dependent papillomas are at very low risk for carcinoma formation, whereas persistent papillomas (those which remain after termination of TPA treatment) are at high risk. The maximum papilloma incidence at 16 weeks was reduced 30% by treatment with 4-NQO in stage III, but the papilloma regression rate was unaffected by urethane or 4-NQO compared to that in acetone-treated mice (Fig. 2*a*). Either 4-NQO or urethane in stage III clearly increased both the rate of carcinoma appearance and the final carcinoma yield relative to TPA or acetone treatment (Fig. 2*b*). The low carcinoma yield in control groups not exposed to TPA in stage II confirms the requirement for a papilloma stage prior to carcinoma formation (Fig. 3). Stage I initiation followed by solvent treatment in stage II and urethane or 4-NQO in stage III produced few papillomas (3 with urethane and 31 with 4-NQO per 40 mouse group) and almost no malignancies (Fig. 3). However, repeated MNNG given to DMBA-initiated or uninitiated CD-1 mice induced carcinomas in over half of the mice (Fig. 4), as we observed in SENCAR mice.

The percent conversion of papillomas to carcinomas

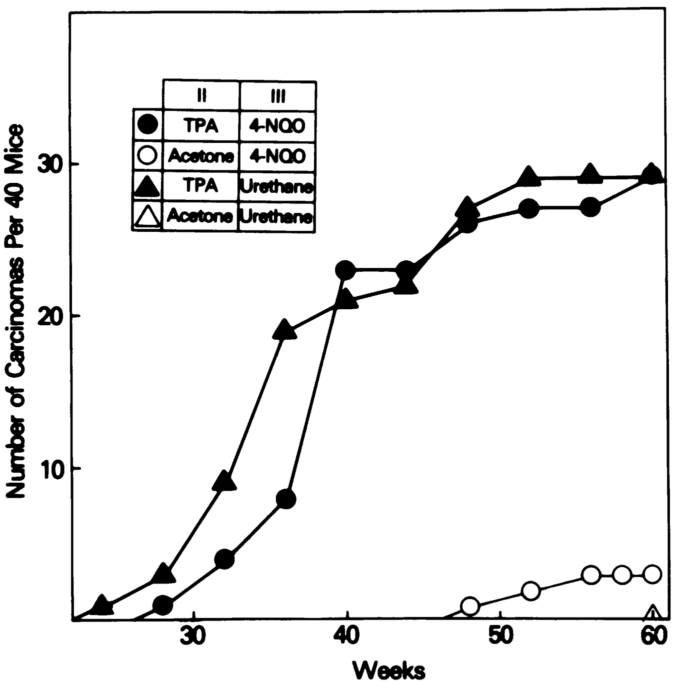

FIGURE 3. Carcinoma incidence in three-stage carcinogenesis in CD-1 mice. In the experiment described in Figure 2, treatment with TPA (●, ▲) is compared to treatment with acetone (○, △) in stage II.

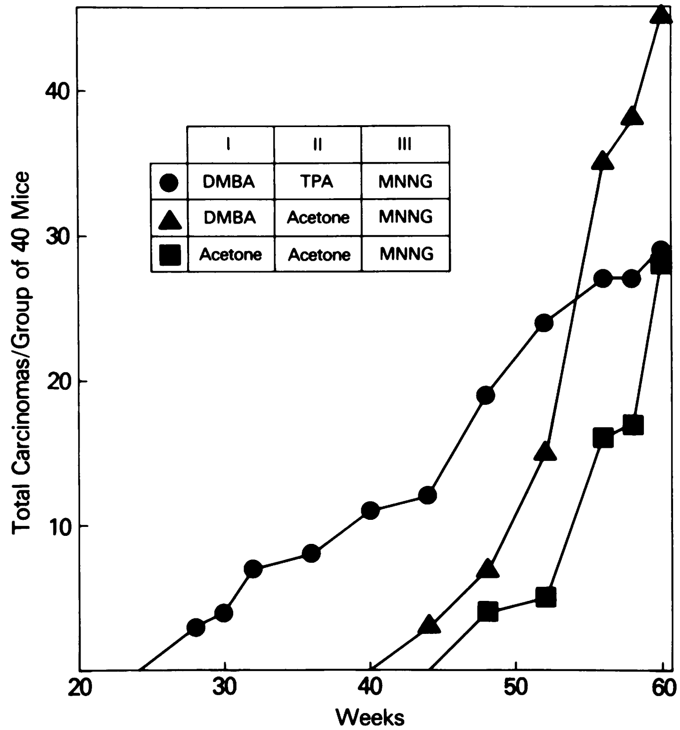

FIGURE 4. Carcinoma incidence in CD-1 mice treated with MNNG in stage III. Mice were treated with DMBA (●, ▲) or acetone (■) solvent in stage I. In stage II, mice received either TPA (10 μg/0.2 mL acetone once weekly for 12 weeks) (●) or 0.2 mL acetone once weekly for 12 weeks (▲, ■). All three groups were treated with MNNG (120 μg/0.2 mL acetone) in stage III.

was calculated at week 60 based on the maximum number of papillomas that developed in each group shown in Figure 2. With continued TPA treatment, a 2.5% malignant conversion rate was found, compared to 3.7% in the acetone control group. The final percent conversion was increased to 5.5% with urethane and to 7.9% with 4-NQO. These percentages are approximately twice those calculated in SENCAR mice (*5*) since the papilloma incidence is about half that found in SENCARs and the carcinoma incidence is comparable. The difference between mice treated with acetone or TPA and mice treated with genotoxic agents in stage III was much more striking at earlier times. At 40 weeks, the percent conversion was 0.75% with acetone, 1.2% with TPA, 4.2% with urethane and 6.3% with 4-NQO. Thus, genotoxic agents in stage III appear to increase the number of carcinomas and also to accelerate their formation.

Malignant Conversion in SENCAR or CD-1 Mice Treated with TPA in Stage III

In contrast to CD-1 mice, continued TPA treatment of initiated SENCAR mice after an optimal course of TPA treatment did not result in a maintenance of a high papilloma incidence (Fig. 5). Papillomas regressed whether mice were exposed to continued TPA or acetone solvent. The mechanism responsible for this apparent absence of TPA-dependent papillomas in SENCAR mice is unclear. As with CD-1 mice, however, the final carcinoma yield was the same with TPA or solvent, although the latent period may be somewhat shorter in the continuous TPA group.

Metastasis of Squamous Cell Carcinomas

In the three-stage carcinogenesis experiment in CD-1 mice described in Figures 2–4, complete autopsies were performed on all animals and possible metastatic lesions were examined histologically. After DMBA initiation, 12 weeks of TPA promotion and either continued TPA or acetone treatment for a further 40 weeks, about 20% of the mice with 1 or more carcinomas also developed metastatic carcinomas (Table 2). With MNNG in stage III, the overall metastasis rate increased to 30%, but with 4-NQO, a metastasis rate of only 15% was found. With acetone, TPA, MNNG, or 4-NQO, a total of 14 metastases were seen in lymph nodes with only 4 in the lungs. Both the pattern and frequency of metastasis were altered in the mice exposed to urethane. Metastases were seen in one-third of the carcinoma-bearing mice, with three in lymph nodes and six in

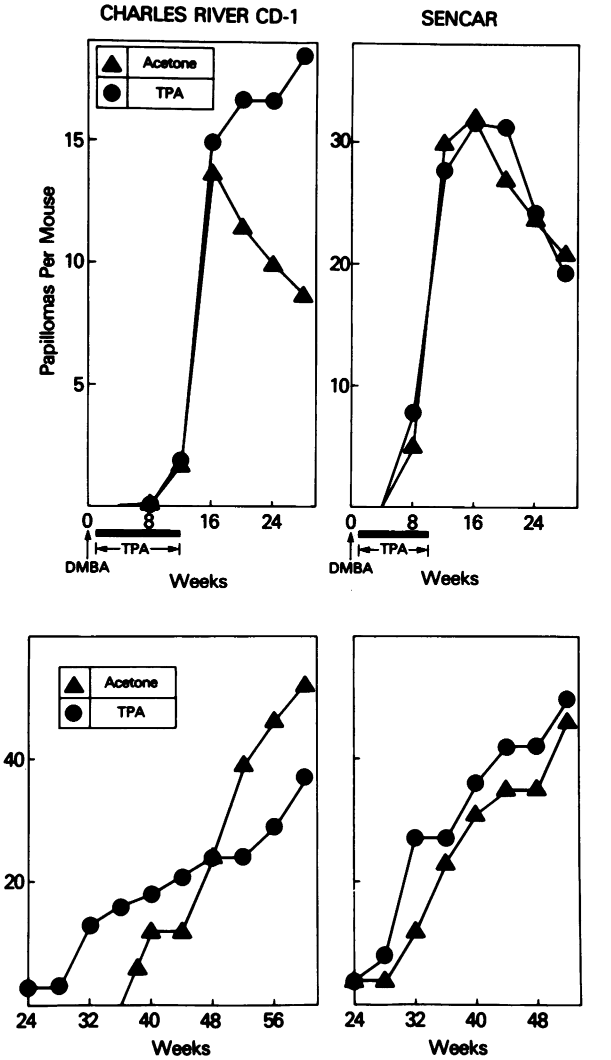

FIGURE 5. Papilloma and carcinoma incidence in papilloma-bearing mice treated with TPA or acetone solvent: comparison of CD-1 and SENCAR mice. Charles River CD-1 mice were initiated with 50 μg DMBA and promoted with 12 weekly applications of 10μg TPA. Beginning at week 13, mice were treated once per week with either (▲) 0.2 mL acetone or (●) 10 μg TPA/0.2 mL acetone. Similarly, papillomas were induced in SENCAR mice by initiation with 20 μg DMBA and promotion by 10 weekly applications of 2.5 μg TPA. Beginning at week 11, mice were treated weekly with either (▲) 0.2 mL acetone or (●) 2.5 μg TPA/0.2 mL acetone.

lungs. The lung squamous cell carcinomas seen after stage III urethane exposure were not primary, urethane-induced lesions since identical urethane treatment of uninitiated mice or initiated mice not treated with TPA in stage II produced no squamous cell carcinomas of the skin or lung. Primary lung adenomas were observed in about half the mice in these two groups as well as in the group that developed metastatic lung tumors.

The overall rate of metastasis appeared to be slightly lower in SENCAR mice treated in stage III with acetone (Table 2). A group treated with TPA in stage III was not included in this experiment, so no data are presented on metastases in SENCAR mice treated long-term with TPA. In contrast to CD-1 mice, stage III exposure to urethane did not affect the number of mice with lung metastases. Although 4-NQO increased the metastatic rate only slightly, MNNG in stage III more than doubled the overall rate of metastasis.

Table 2. Frequency and distribution of metastases in mice bearing skin squamous cell carcinomas.

Stage III treatment	% of carcinoma-bearing mice with metastases[a]: Total (one or more sites)		Lymph node		Lung	
	CD-1	SENCAR	CD-1	SENCAR	CD-1	SENCAR
Acetone	16.7	11.1	16.7	5.6	11.1	11.1
TPA	23.1	—	23.1	—	0	—
MNNG	30.0	26.3	25.0	15.8	5.0	15.8
Urethane	33.3	11.1	12.5	0	25.0	11.1
4-NQO	15.0	19.0	15.0	9.5	5.0	9.5

[a] Groups of 40 female CD-1 mice were initiated with 50 μg DMBA (stage I) and promoted with 12 weekly applications of 10 μg TPA (stage II). In SENCAR mice, groups of 30 females were initiated by 20 μg DMBA and promoted with 10 weekly applications of 2 μg TPA. The indicated stage III treatments once weekly from weeks 13 to 52 in CD-1 mice and weeks 11 to 40 in SENCAR mice were as follows: acetone, 0.2 mL; TPA, 10 μg; MNNG, 120 μg; urethane, 20 mg IP; 4-NQO, 250 μg. Some animals developed metastases in both lymph nodes and lungs. Between 13 and 24 carcinoma-bearing mice were examined in each group. Squamous cell carcinomas in the lungs or lymph nodes were observed only in mice that bore skin squamous cell carcinomas.

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